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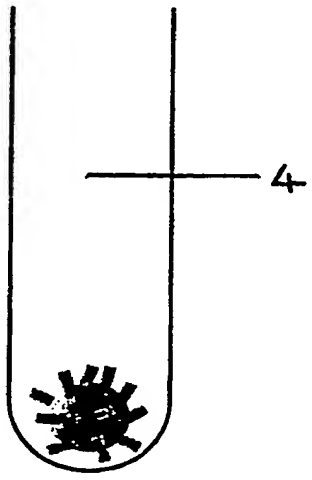
(54) **Title:** DIAGNOSIS OF NEURO–DEGENERATIVE DISORDERS

(57) **Abstract**

The diagnostic method comprises concentrating a protein associated with the neuro–degenerative disorder in a sample of a body fluid taken from the animal. The concentration is carried out by contacting the sample with a solid, non–buoyant particulate material having free ionic valencies. The resulting protein associated with the neuro–degenerative disorder concentrated on the particulate material is then monitored.



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Diagnosis of Neuro-Degenerative Disorders

The present invention relates to diagnosis of neuro-degenerative disorders in humans and animals.

Neuro-degenerative disorders include non-transmissible diseases such as Alzheimer's disease and multiple sclerosis, and spongiform encephalopathies.

Spongiform encephalopathies, such as Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker Syndrome (GSS) and Kuru in humans; scrapie in sheep and goats and bovine spongiform encephalopathy (BSE) in cattle, mink and cats are all transmissible (infective) neuro-degenerative disorders implicating vacuolation of neurons.

At present, the most reliable method of detecting an encephalopathy is histologically, especially by electron microscopy, but this requires brain tissue removed following autopsy of the dead victim. Although neurological examination and electro-encephalographs (EEG) can provide accurate diagnosis in many cases of encephalopathy, there is an urgent need for a definitive test during life, one which can detect the disease during its early stages and which is non-intrusive.

It is therefore the aim of the present invention to provide a means for the rapid and early diagnosis of encephalopathy using non-intrusive means such as a urine test.

The protein associated with for example the neuro-degenerative disorder CJD is thought to be a particle termed a "nemavirus". In contrast to the morphology of a common virus, which has a two layer structure of nucleic acid protected by an outer coat, the nemavirus particle has an unusual three layer structure which comprises:

1. a protein core,
2. single stranded DNA, and
3. a protein coat

The single stranded DNA is sandwiched between the protein core and the protein coat. Single stranded DNA from scrapie has been partly sequenced and contains a palindromic repeat sequence TACGTA. The scrapie-specific nucleic acid is single stranded DNA and includes the sequence $(TACGTA)_n$ where n is at least 6. The basic six unit of this repeat sequence is palindromic, in the sense that a complementary DNA would have the same TACGTA sequence when read in the 5' to the 3' direction. The full length sequence of the DNA is not known, but it is suspected that n is very much larger than 6, perhaps of the order of 20 to 30. Although the DNA sequence is scrapie-specific, BSE, scrapie, CJD and other encephalopathies are thought to result from the same protein associated with the neuro-degenerative disorder transferred to another species. It is therefore believed that the TACGTA palindromic sequence appears in all known spongiform encephalopathies and possibly others.

The protein coat has not yet been characterised. The protein core comprises the protease-resistant protein (PrP) which is termed a "prion". A prion is encoded by a cellular gene of the host and is thought to contain little or no nucleic acid. However, the cellular form of the prion protein is modified into protease-resistant protein (PrP), by an accessory protein, "Nemo Corrupta" coded by single stranded DNA (PESM, 212, 208-224, (1996). This feature distinguishes prions sharply from virions. To date, no prion-specific nucleic acid which is required for transmission of disease has been identified.

Virus-like nemaviruses are tubulofilamentous particles in shape, typically 23-26 nm in diameter. They are consistently detected in the brains of all known spongiform encephalopathies. These particles have a core of prion in a rod-like form; the prion rods being also termed scrapie-associated fibrils (SAF). Over the core is a layer of DNA, removable by DNAse; above the core is an outer protein coat which is digestible by a protease.

It would be desirable to have a method of diagnosis based on nucleic acid identification or on the core structure of the nemavirus protease-resistant protein in a living human or animal. Such methods have been suggested where a probe of DNA derived from the gene sequence coding for a prion protein are used. However, since it is well known that prion protein is encoded by a normal chromosomal gene found in all mammals, including those affected by encephalopathies, the above work has not gained acceptance. PCT Patent Application W089/11545 (Institute for Animal Health Ltd) purports to describe a method of detection of scrapie susceptibility by use of a restriction fragment length polymorphism (RFLP) linked to the so called Sinc gene associated with short incubation times of sheep infected by scrapie. The RFLP is said to be located in a non-coding portion associated with the gene for the prion. At best, this method would detect only sheep with the short incubation time characteristic. Hitherto, methods of diagnosis based on nucleic acid identification have not been very successful or are likely to be unsuccessful, since an encephalopathy specific nucleic acid has eluded detection despite numerous attempts.

In human CJD cases, the protein associated with the neuro-degenerative disorder has been consistently shown by titration studies to be present in blood. Although the protein associated with the neuro-degenerative disorder is present in urine of CJD cases, there is no known technique of diagnosis based on urine.

I have now developed a diagnostic method, which may be quantitative or semi-quantitative, in which some estimate is made of the amount of the protein associated with the neuro-degenerative disorder from a urine specimen which can be collected from the live animal.

I have previously described, in patent number 2258867, a method for the diagnosis of encephalopathy using animal tissue. This method includes the use of a scrapie-specific nucleic acid, part of which can be labelled and used as an oligonucleotide

probe in a hybridisation assay. Alternatively, a sequence from the scrapie-specific nucleic acid is used as a primer in a polymerase chain reaction to make sufficient quantities to allow detection by a restriction fragment length method.

The method according to the invention for the diagnosis of a neuro-degenerative disease(such as encephalopathy), in an animal, comprises concentrating a protein associated with the neuro-degenerative disorder (such as a nemavirus) in a sample of a body fluid (such as urine) taken from the animal, the concentration being by contacting the sample with a solid, non-buoyant particulate material having free ionic valencies; and monitoring the resulting protein associated with the neuro-degenerative disorder concentrated on the particulate material.

The concentration of the protein associated with neuro-degenerative disorders takes place as a result of aggregation thereof on the surface of the particulate material.

As indicated, the nemavirus or protein associated with neuro-degenerative disorders is concentrated from a body fluid, such as urine, using a solid non-buoyant particulate material, a preferred example of which is calcium phosphate. Calcium phosphate is widely used in transformation experiments to allow the introduction of DNA into a living cell, wherein it causes the precipitation of DNA. However, it has not been previously suggested for the purpose of concentrating a protein associated with neuro-degenerative disorder in a diagnostic sample of urine or the like.

The particulate material is preferably in the form of granules. Part of the protein associated with the neuro-degenerative disorder (in the case of spongiform encephalopathies) or amyloid precursor protein APP (in the case of a non-transmissible neuro-degenerative disease, such as Alzheimer's and basic myelin protein oligocyte for multiple sclerosis) is thought to bind to the surface of the granules.

The steps leading to the concentration of the protein associated with a neurodegenerative disorder from a sample of urine typically comprise:

(a) collecting and centrifuging a sample of urine from an infected animal;

(b) collecting the supernatant produced following centrifugation of the sample of urine;

(c) adding a buffer and a solid, non-buoyant particulate material having free ionic valencies (such as calcium phosphate granules) to the supernatant;

(d) centrifuging the resulting mixture of buffer, particulate material and supernatant;

(e) collecting particulate material following centrifugation;

(f) adding a buffer to the particulate material;

(g) centrifuging the mixture of buffer and particulate material;

(h) collecting the particulate material;

(i) adding a buffer to said particulate material;

(j) centrifuging a mixture of the buffer and the particulate material; and

(k) collecting the particulate material which will contain the protein associated with the neuro-degenerative disorder.

The sample of urine or the like can be concentrated 100 fold or more using calcium phosphate or other non-buoyant particulate material in the method according to the invention; the concentrated urine can then be used in several ways to allow diagnosis of neuro-degenerative disorder.

According to one aspect of the present invention, the concentrated sample of urine can be used for the detection of tubulofilamentous particles using electron microscopy. In such a method, a grid is brought into contact with the sample of concentrated urine and then the grid is fixed and stained. This allows the tubulofilamentous particles that are characteristic of the nemavirus or protein associated with the neuro-degenerative disorder to be visualized by electron microscopy.

Diagnosis of encephalopathy can alternatively be carried out by means of, for example, an enzyme-linked immunosorbent assay (ELISA). The ELISA technique can be automated to provide a semi-quantitative result. The calcium phosphate for the concentration of the nemavirus would be included as part of an ELISA kit. Such a kit according to the invention preferably further comprises a blocking buffer, an antibody to PrP and an antibody conjugate. A kit according to the invention preferably comprises:

(a) a solid, non-buoyant particulate material having free ionic valencies (such as calcium phosphate) in a form capable of complexing with protein present in a body fluid;

(b) a blocking buffer capable of complexing with any of said particulate material not complexed with said protein;

(c) a first antibody material capable of complexing with said complexed protein; and

(d) a further antibody which is capable of complexing with said first antibody.

When the neuro-degenerative disorder is a spongiform encephalopathy, an antibody to PrP may be added which will bind to the protein associated with the neuro-degenerative disorder on the surface of the particulate material. This is generally followed by a second antibody which will bind to the previous antibody, the second antibody being conjugated to a marker enzyme to allow detection of the protein associated with the neuro-degenerative disorder.

The use of calcium phosphate in the concentration of the protein associated with the neuro-degenerative disorder and the subsequent detection using an ELISA method is shown schematically in Figures 1 to 6 of the accompanying drawings, which are by way of example only, and in which:

Figure 1 shows a reaction vessel 1, having therein an exemplary calcium phosphate granule 2 and a protein 3 associated with a neuro-degenerative disorder;

Figure 2 shows the protein 3 associated with the neuro-degenerative disorder concentrated on the surface of the calcium phosphate granule 2;

Figure 3 shows the unbonded sites on the surface of the calcium phosphate granule 2 blocked on the addition of blocking buffer (such as milk) 4;

Figure 4 shows the addition of a first antibody against the protein associated with a neuro-degenerative disorder 5;

Figure 5 shows binding of the first antibody 5 to the protein 3 associated with a neuro-degenerative disorder which is still bonded to the surface of the calcium phosphate granule 2;

Figure 6 shows antibody detection using a second antibody 6 conjugated to a marker enzyme such as horseradish peroxidase or alkaline phosphatase; and

Figure 7 is a photograph of a stained blot obtained in an exemplary diagnostic method according to the invention.

Another method for the diagnosis of encephalopathy from the concentrated urine sample is to amplify the DNA in the sample by using a polymerase chain reaction (PCR). In a preferred method, the palindromic oligonucleotide described above is used to amplify the sample DNA. Such oligonucleotides will not normally be longer than 200 nucleotides, even when used as probes; generally, they are likely to be very much shorter. Thus, for PCR purposes they are unlikely to comprise more than 24 nucleotides of the palindrome, plus an optional 5'-end or tail of (say) 8 to 20 nucleotides, making 32 to 44 nucleotides in all. The PCR will yield a product in the form of DNA of varying lengths containing the palindromic sequence. This can preferably be analysed by a method relying on restriction by an enzyme.

The PCR product will produce bands of various molecular weights. In some instances the encephalopathy-specific DNA will be primed near its 3'-end, which will generate multiple copies of large molecules. The PCR product may be divided into two portions, of which the first may be run on a resolving gel to show a band of high molecular weight associated with the

encephalopathy-specific DNA, the second portion being restricted with a restriction enzyme which cuts the palindromic sequence. This restriction will severely reduce the length of the longer DNA and eliminate certain other bands of shorter DNA altogether. Multiple restrictions of TACGTA will produce many bands of molecular weight too low to be detected. Restricted product can be compared with the unrestricted product, whereby disappearance of longer lengths of DNA upon restriction indicates the presence of the encephalopathy-specific DNA in the sample.

Examples of suitable restriction enzymes are SnaBI and AccI, which cut between the C and G of TACGTA and Bst11071 which cuts between A and T of one TACGTA sequence and the next TACGTA sequence. Such enzymes recognise the six-base sequence and leave blunt ends.

The sample of urine or other body fluid containing the concentrated protein associated with the neuro-degenerative disorder can be used in a further assay for the diagnosis of encephalopathy, using a hybridisation method. In the hybridisation method, the sample of urine or the like, containing the protein associated with the neuro-degenerative disorder, can be used as it is, or preferably, it may be amplified before use, for example, using a PCR method. The hybridisation probe is preferably from 16 to 100 nucleotides long, especially about 40 nucleotides long. The hybridisation assay can be carried out in a conventional manner; Southern blotting is preferred. For use in a hybridisation assay, the oligonucleotide will normally be used in a labelled form, labelling being by any appropriate method such as radiolabelling, for example, by ^{32}P or ^{35}S, or by biotinylation (which can be followed by reaction with labelled avidin). However, it is also possible to use an unlabelled oligonucleotide as a probe provided that it is subsequently linked to a label. For example, the oligonucleotide could be provided with a poly-C tail which could be linked subsequently to labelled poly-G.

An alternative method for the diagnosis of encephalopathy is using a protein blotting method (Western blotting) which comprises detecting the protein of interest on the surface of a membrane (such as nitrocellulose) and detection of the protein using antibody technology.

Methods and Materials

1. Purification of a Protein associated with a Neuro-Degenerative Disorder from a sample (for example urine)

A sample of urine was collected from an animal suspected of being infected. The urine sample was centrifuged and the supernatant collected. A suitable buffer and calcium phosphate granules were then added to the supernatant. This mixture of urine supernatant, buffer and calcium phosphate was allowed to rest at room temperature (with regular mixing) for at least ten minutes. The mixture was then centrifuged and the calcium phosphate granules collected. A suitable buffer was then added to the calcium phosphate granules followed by a further centrifugation step. The calcium phosphate granules were collected and the above addition of buffer and centrifugation step was repeated a further two times. The calcium phosphate granules were collected for the detection of a possible protein associated with a neurodegenerative disorder using any of methods 2,3,4,5,6 and 7 as follows.

2. Enzyme Linked Immunosorbent Assay

The calcium phosphate granules obtained following the above purification stage were used.

A suitable blocking buffer (for example milk) was added to the calcium phosphate granules and the solution was left mixing for at least sixty minutes. The solution was then centrifuged and the supernatant discarded. To the calcium phosphate granules that remain some phosphate buffered saline (PBS) containing Tween

20 was added and this was followed by a further centrifugation step. The above PBS-Tween 20 wash step was repeated at least four times. A first antibody was then added to the calcium phosphate granules. This was left to stand for at least 60 minutes with mixing at regular intervals. PBS-Tween 20 was added and followed by a centrifugation step. The supernatant was discarded and the PBS-Tween 20 wash step repeated at least four times. A second antibody, (one conjugated to a marker enzyme) was then added to the calcium phosphate granules and left mixing for at least sixty minutes. PBS-Tween 20 was then added followed by a centrifugation step. The supernatant was discarded and the wash step repeated with PBS-Tween 20 at least four times.
A substrate suitable for detection of the marker enzyme on the second antibody was then added. This was left to stand for at least twenty minutes and the reaction stopped by addition of a suitable reagent, such as concentrated sulphuric acid. Following centrifugation, the supernatant was collected and read photometrically at a suitable wavelength.

3. Preparation of grids for electron microscopy.

The calcium phosphate granules obtained following the purification steps outlined in method 1 were used.
Ethylenediaminetetraacetic acid (EDTA) was added to the calcium phosphate granules and mixed until a clear solution was produced. A carbon-coated grid was lowered into tubes containing some distilled water making sure the carbon/Formvar film was facing upwards. For each specimen at least two grids were prepared using the clear solution which was then transferred into the tube whilst gently rinsing off the distilled water. The grids were then centrifuged horizontally. After the centrifugation step sodium dodecyl sulphate was added and the grids transferred into distilled water. The grids were then washed several times in distilled water. The water was then momentarily replaced with glutaraldehyde containing ruthenium red. This solution was then rinsed out with distilled water and the grids were then

momentarily introduced to a solution of osmic acid containing ruthenium red. The grids were again rinsed several times with distilled water. After the final wash of water with a drop of phosphotungstic acid the grids were dried and examined under an electron microscope.

4. Polymerase Chain Reaction

Again the calcium phosphate granules obtained following the purification stage (method 1) were used.

EDTA was added to the calcium phosphate granules until a clear solution was produced. Some of this clear solution was taken and incubated with proteinase K for at least one hour at 55°C. The proteinase K was then heat inactivated at 95°C and the solution used as a template in a polymerase chain reaction (PCR). A dNTP mix, primers, a buffer and AmpliTaq DNA polymerase were then added to the reaction mixture. Thirty cycles of PCR were carried out comprising a denaturation stage, annealing of primers and an extension stage. The PCR product was then cut with the restriction enzyme SnaB1. Cut and uncut PCR product was then analysed using electrophoresis and the fragments visualised on the agarose gel after staining with ethidium bromide.

5. Protein Blotting for immunoassay

Bio-Dot apparatus was used for the immunoblotting procedure. Nitrocellulose membranes were pre-wetted in Tris saline buffer (TSB) prior to placing in the bio-dot apparatus. After re-hydrating the membrane the wells of the apparatus were filled with antigen. The antigen solution being the clear solution produced on mixing the calcium phosphate granules from method 1 with EDTA). The entire antigen sample was allowed to filter through the membrane. After the antigen samples had completely drained from the apparatus the Tris saline buffer (TSB) was added and the liquid allowed to filter through. Blocking solution was then added to each well and the liquid allowed to filter through the apparatus. Tween-tris saline buffer (TTSB) wash solution was

added to the apparatus and the flow valve adjusted to produce a vacuum to pull the wash solution through the membrane. The vacuum was then disconnected and a first antibody solution added to each sample well. The solution was allowed to filter through the membrane and the vacuum was re-applied to remove any excess liquid from the sample wells. TTSB wash solution was then added and pulled through the membrane with the aid of a vacuum. The wash process was then repeated three times.

Conjugated antibody solution was added to each well and the liquid allowed to filter through. TTSB wash solution was then added to each well and the solution pulled through the membrane with the aid of a vacuum. This wash process was repeated twice. The membrane was removed and placed in the colour development vessel. The membrane was then removed and washed with TSB to remove excess Tween 20. The membrane was then incubated in a suitable substrate until the development of spots were seen. After this time the membrane was rinsed in distilled water and photographed.

6. Southern Blotting

A solution obtained from the calcium phosphate granules (method 1) was taken and concentrated NaOH and DMSO added. The solution was mixed and heated and then cooled down to room temperature after which concentrated ammonium acetate was added. Nitrocellulose membrane was then wetted in 6XSSC and the bio-dot apparatus assembled. The DNA sample was applied and allowed to filter through the membrane. After the sample had filtered 2XSSC was added to each well and vacuum applied to remove the liquid. The blot membrane was removed and washed in 2XSSC. The nitrocellulose membrane was then baked under vacuum before hydridisation.

7. Western Blotting

The calcium phosphate granules obtained following the purification steps outlined in method 1 were used. Sodium dodecyl sulphate containing proteinase k was then added to the calcium phosphate granules and the mixture incubated for at least 60 minutes at 55°C. β-mercaptoethanol was added after the incubation period and the mixture was then boiled. Following this polyacrylamide gel electrophoresis was carried out. Proteins on the polyacrylamide gel were then transferred to a nitrocellulose membrane. The membrane was air dried and then washed in tris buffered saline. Any unabsorbed sites were then blocked using heat inactivated horse serum and goat milk. A first antibody made up in tris-buffered saline containing Tween 20 and milk was then applied to the membrane which was left to incubate for at least one hour. The membrane was then washed several times. A second antibody conjugated to a marker enzyme (which was also made up in a solution of tris-buffered saline containing Tween 20) was then applied to the membrane. This was left to incubate for at least 60 minutes and then washed in a solution of tris buffered saline to remove excess Tween 20. The membrane was then incubated in a suitable substrate until the development of bands were seen. After this time the membrane was rinsed in distilled water and photographed.

In an exemplary method, beta-amyloid protein (APP) was concentrated from urine specimens of patient having Alzheiemer's by the method described above and a Western blot performed. The resulting blot, stained by APP-antibody 369, is shown in Figure 7 of the accompanying drawings. Positive results are seen in lane 0, control APP, lanes 1,3,4,6,9,10,11 and M from specimens from Alzheimer's patients.

Lane 3 is control and lane 7 relates to an assay for specimens from patients with Parkinson's disease.

Claims:

1. A method for the diagnosis of neuro-degenerative disorders in an animal using a sample of body fluid from the animal, which method comprises concentrating a protein associated with a neuro-degenerative disorder in said sample, said concentration being by contacting said sample with a solid, non-buoyant particulate material having free ionic valencies; and monitoring the resulting protein associated with a neuro-degenerative disorder concentrated on said particulate material.

2. A method according to claim 1, wherein the particulate material comprises calcium phosphate in granular form.

3. A method according to claim 1 or 2, wherein said concentrated protein is monitored using electron microscopy.

4. A method according to claim 1 or 2, wherein said concentrated protein is monitored using an enzyme linked immunosorbent assay (ELISA).

5. A method according to claim 4, in which a first antibody is added to said concentrated protein associated with a neuro-degenerative disorder so as to permit said first antibody to complex with said concentrated protein.

6. A method according to claim 5, wherein a second antibody which is conjugated to a marker enzyme is added to said complexed protein so as to permit said second antibody to complex to said first antibody.

7. A method according to claim 1 or 2, wherein said concentrated protein in said urine sample is amplified using a polymerase chain reaction and then monitored by a restriction fragment length method.

8. A method according to claim 1 or 2, wherein said concentrated protein in said urine sample is used in a hybridisation reaction and then monitored using Western blotting.

9. A kit for carrying out an ELISA reaction, the kit comprising:

(a) a solid, non-buoyant particulate material having free ionic valencies (such as calcium phosphate) in a form capable of complexing with protein associated with a neuro-degenerative disorder present in a body fluid;

(b) a blocking buffer capable of complexing with any of said particulate material not complexed with said protein;

(c) a first antibody material capable of complexing with said complexed protein; and

(d) a further antibody which is capable of complexing with said first antibody.

10. A method for concentrating a protein associated with neuro-degenerative disorder from a sample of urine which comprises the following steps:

(a) collecting and centrifuging a sample of urine from an infected animal;

(b) collecting the supernatant produced following centrifugation of said sample of urine;

(c) adding a buffer and a solid, non-buoyant particulate material having free ionic valencies (such as calcium phosphate granules) to said supernatant;

(d) centrifuging the resulting mixture of said buffer, said particulate material and said supernatant;

(e) collecting said particulate material following centrifugation;

(f) adding a buffer to said particulate material;

(g) centrifuging said mixture of said buffer and said particulate material;

(h) collecting said particulate material;

(i) adding a buffer to said particulate material;

(j) centrifuging a mixture of said buffer and said particulate material; and

(k) collecting supernatant which will contain the protein associated with neuro-degenerative disorder.

FIGURE 1

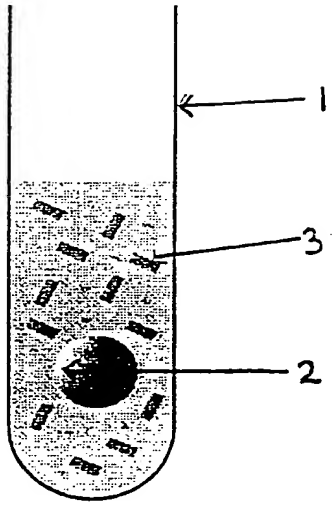

FIGURE 2

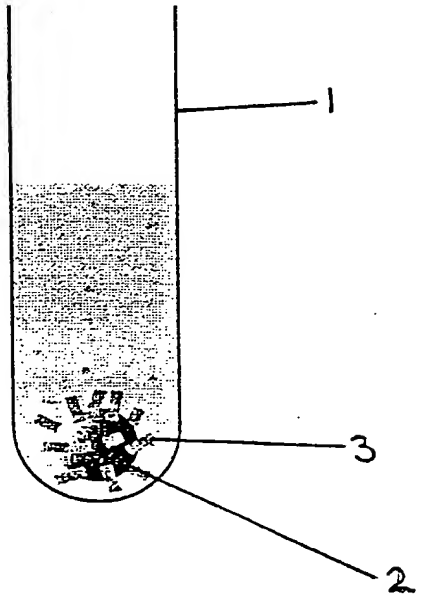

FIGURE 3

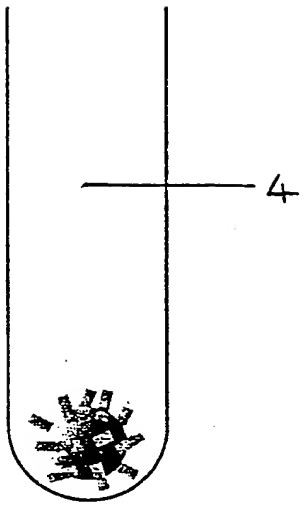

FIGURE 4

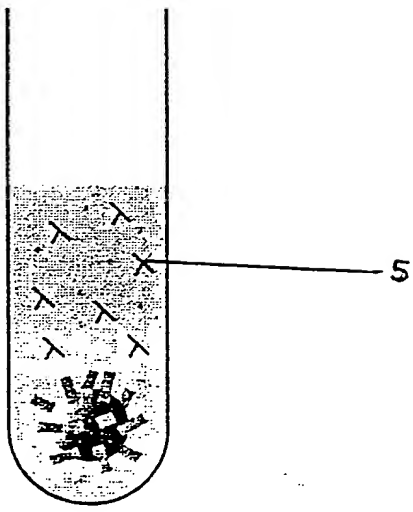

FIGURE 5

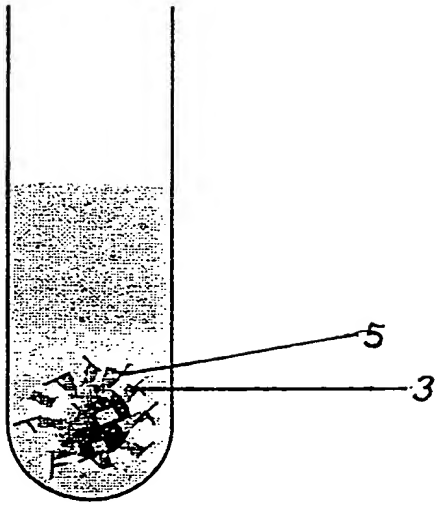

FIGURE 6

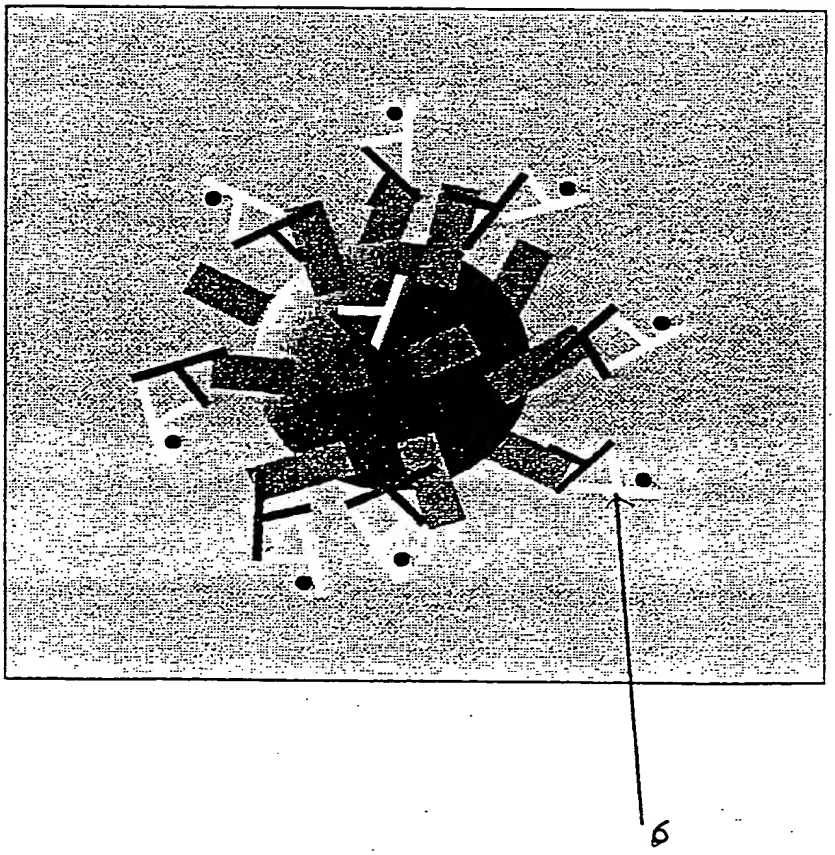

FIGURE 7